Optimizing Phosphorus Characterization in Animal Manures by Solution Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT

A procedure involving alkaline extraction and solution ^{31}P nuclear magnetic resonance (NMR) spectroscopy was developed and optimized for the characterization of P in animal manures (broiler, swine, beef cattle). Inclusion of ethylenediaminetetraacetic acid (EDTA) in the alkaline extraction solution recovered between 82 and 97% of the total P from the three manures, which represented a significant improvement on recovery in NaOH alone. Low concentrations of paramagnetic ions in all manure extracts meant that relatively long delay times (>5 s) were required for quantitative analysis by solution ^{31}P NMR spectroscopy. The manures contained inorganic orthophosphate, orthophosphate monoesters, orthophosphate diesters, and inorganic polyphosphates, but results were markedly influenced by the concentration of NaOH in the extractant, which affected both spectral resolution and the apparent P composition of the extracts. For example, extraction of swine manure and broiler litter with 0.5 *M* NaOH + 50 m*M* EDTA produced remarkable spectral resolution that allowed accurate quantification of the four signals from phytic acid, the major organic P compound in these manures. In contrast, more dilute NaOH concentrations produced considerable line broadening that obscured individual signals in the orthophosphate monoester region of the spectra. Spectral resolution of cattle manure extracts was relatively unaffected by NaOH concentration. Improvements in spectral resolution of more concentrated NaOH extracts were, however, compromised by the disappearance of phospholipids and inorganic polyphosphates, notably in swine and cattle manure extracts, which indicated either degradation or a change in solubility. The optimum extraction conditions will therefore vary depending on the manure type and the objectives of the study. Phytic acid can be accurately quantified in swine manure and broiler litter by extraction with 0.5 *M* NaOH + 50 m*M* EDTA, while a more dilute NaOH concentration should be used for complete P characterization or comparison among different manure types.

There is currently great interest in the P composition of animal manures, because long-term manure application to agricultural land can lead to soil P accumulation and an acceleration of P transfer in runoff to water bodies (Sims et al., 2000). This can contribute to eutrophication, and numerous examples of water quality impairment associated with P pollution from animal operations now exist (e.g., Burkholder et al., 1992; Environment Protection Authority, 1995). To address this, strategies to decrease P in manures are being widely adopted. Such strategies are based on increasing the availability of grain P to the animal, because phytic acid (*myo*-inositol hexa*kis*phosphate), the dominant P compound in most cereal grains, cannot be digested by monogastric animals (Taylor, 1965). Strategies to increase the availability of grain P to monogastrics include the isolation of mutant grains having low phytic acid concentrations (Raboy et al., 2000), and supplementation of animal diets with microbial phytase enzymes that catalyze the hydrolysis of phytic acid in the gut (Simons et al., 1990). Both techniques allow inorganic P supplements to be minimized.

The development of robust techniques for identifying P compounds in animal manures is a fundamental prerequisite to understanding the effects of dietary manipulations on animals and the environment. The first comprehensive evaluation of manure P composition was reported by Peperzak et al. (1959), who used a fractionation procedure to determine that phytic acid (measured as acid-soluble organic P) dominated the organic P component of a wide range of manures, with relatively small proportions of phospholipids and DNA. Comparable results were obtained by subsequent authors using similar procedures (Gerritse and Vriesema, 1984; Barnett, 1994). Various chromatographic techniques are routinely employed for the specific determination of phytic acid in feeds (Phillippy and Bland, 1988; Dušková et al., 2000; Raboy et al., 2000), but are rarely applied to animal manures because phosphates exist in complexes with mineral and organic compounds that confound separation by conventional procedures. Sample analysis by chromatography is rapid compared with spectroscopic techniques, but errors can arise through poor recoveries from chromatography columns, matrix interference with standards, and misidentification of compounds (Kemme et al., 1999; Turner et al., 2002). In most cases, a secondary technique, such as solution ^{31}P NMR spectroscopy, is required to identify the separated fractions (Kemme et al., 1999; Raboy et al., 2000). Recently, an enzyme hydrolyzable P technique was employed to characterize functional P groups in chemically extracted fractions of swine and cattle manures (He and Honeycutt, 2001). This relatively low-cost method has promise as a routine laboratory procedure for manure P characterization, although large proportions of the total P remain unidentified.

Spectroscopic techniques are less frequently employed for manure P speciation. The value of solid-state ^{31}P NMR spectroscopy remains limited at present due to poor spectral resolution (Frossard et al., 2002), but solution ^{31}P NMR spectroscopy has numerous benefits over chromatographic and other procedures, and potentially offers the most convenient method of P characterization. In particular, it allows the simultaneous identification of multiple P compounds in the complex matrices

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Abbreviations: NMR, nuclear magnetic resonance.

of manure extracts with minimal sample handling (Condron et al., 1997). The technique has been widely applied to soils during the previous two decades, and was advanced recently by improvements in the extraction procedure, signal identification, and the understanding of compound degradation during extraction and analysis (Cade-Menun and Preston, 1996; Makarov et al., 2002; Turner et al., 2003a). Importantly, the inclusion of EDTA in the alkaline extraction solution markedly improves P recovery from soils (Bowman and Moir, 1993), but this has not been tested for animal manures.

Solution ^{31}P NMR spectroscopic procedures have been developed for quantification of phytic acid in food (O'Neill et al., 1980), animal feed (Kemme et al., 1999), and sewage sludge (Hinedi et al., 1989). Of the few studies using solution ^{31}P NMR spectroscopy to analyze animal manure, Leinweber et al. (1997) identified orthophosphate monoesters and diesters in NaOH extracts of swine slurry, while Crouse et al. (2000) characterized functional P groups in NaOH–EDTA extracts of turkey litter. Spectral resolution was relatively poor in these studies, and this represents perhaps the greatest limitation of the technique. In particular, poor resolution in the orthophosphate monoester region of the spectra often precludes accurate quantification of phytic acid, the dominant organic P compound in most manures.

Given the current interest in the P composition of animal manures, there is an urgent requirement for a comprehensive study of their analysis by solution ^{31}P NMR spectroscopy. The aim of this work was to address this by investigating the optimal extraction conditions for different types of animal manures, the potential degradation of P compounds during extraction and analysis, and the optimal NMR machine parameters for quantitative spectroscopy. The ultimate aim was to recommend a standard procedure for the extraction and analysis of animal manures by solution ^{31}P NMR spectroscopy.

MATERIALS AND METHODS

Phosphorus Recovery from Manures

Three manure samples were obtained: a swine manure (grain fed) and a beef-cattle manure (pasture-fed) from commercial farms in southern Idaho, and a broiler litter (mixture of broiler manure and sawdust bedding) from an experimental farm in Delaware. The samples were immediately frozen at −80°C, lyophilized (approximately 4 d), and ground to pass a 500-μm sieve. Dry matter contents were 25% for the swine manure, 14% for the cattle manure, and 84% for the broiler litter. Total elements were determined by microwave digestion in concentrated HNO_3 and H_2O_2 (USEPA, 1996), with detection by inductively coupled plasma–atomic emission spectrometry (ICP–AES) (Table 1).

Table 1. Elemental analysis of manures used in the study determined by nitric acid–sodium peroxide microwave digestion and inductively coupled plasma–atomic emission spectrometry (ICP–AES) detection.†

Element	Broiler litter	Cattle manure	Swine manure
	mg g^{-1} dry manure		
Al	0.47 ± <0.01	1.53 ± 0.06	0.22 ± 0.01
Ca	20.6 ± 0.32	15.9 ± 0.12	11.9 ± 0.07
Fe	0.86 ± 0.02	1.62 ± 0.03	1.13 ± 0.05
Mn	0.53 ± 0.01	0.22 ± <0.01	0.11 ± <0.01
P	15.96 ± 0.04	4.94 ± 0.06	14.62 ± 0.19

† Values are means ± standard deviations of three replicate digests.

The influence of EDTA on P recovery in alkaline solution was investigated by extracting manures with varying concentrations of NaOH (0, 0.15, 0.25, and 0.50 *M*) and EDTA (0, 10, 25, and 50 m*M*) for 4 h. The effect of extraction time on P recovery was investigated by extracting manures in a solution containing 0.25 *M* NaOH and 50 m*M* EDTA for varying times from 1 to 16 h. In both cases, 1.00 ± 0.01 g samples of manure were mixed with 20 mL solution and shaken horizontally in 50-mL centrifuge tubes at 20°C. Extracts were then centrifuged at 10 000 × *g* for 30 min, and aliquots (5 mL) diluted 20-fold and analyzed for total P and cations (Al, Ca, Fe, Mn) by ICP–AES. Reactive P, which approximates to inorganic orthophosphate, was determined by molybdate colorimetry (Murphy and Riley, 1962) after an additional fivefold dilution (EDTA interferes with the reaction at concentrations of >2 m*M*). Unreactive P, which includes organic P and inorganic polyphosphates, was calculated as the difference between total P and reactive P. For comparison with standard acid extraction, manures were also extracted with 1 *M* HCl for 1 h and analyzed for P fractions as described above.

Solution Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy

Manures (2.00 ± 0.01 g) were extracted with 40 mL of NaOH–EDTA for 4 h at 20°C. Three extraction solutions were used, containing different concentrations of NaOH (0.15, 0.25, and 0.50 *M*) with a constant concentration of 50 m*M* EDTA. Extracts were centrifuged and aliquots (2 mL) taken for chemical analysis as described above. The remainder of the extracts was then frozen rapidly at −80°C, lyophilized, and ground to a fine powder. Immediately before NMR spectroscopy, each freeze-dried extract (approximately 100 mg) was redissolved in 0.9 mL of 1 *M* NaOH and 0.1 mL of D_2O (for signal lock) and transferred to a 5-mm NMR tube. The pH of the redissolved samples varied slightly depending on the initial extractant concentration, with averages for 0.15, 0.25, and 0.50 *M* NaOH extracts of the three manures being 13.71 ± 0.06, 13.78 ± 0.07, and 13.99 ± 0.06, respectively.

Solution ^{31}P NMR spectra were obtained using a Bruker (Billerica, MA) Avance DRX 500 MHz spectrometer operating at 202.456 MHz for ^{31}P and 500.134 MHz for ^{1}H. We used a 5-μs pulse (45°), a delay time of 5.0 s, an acquisition time of 0.8 s, and broadband proton decoupling for all samples. The number of scans required to give an acceptable signal-to-noise ratio varied among the manures depending on total P concentration and extract properties, being 1500 to 5500 for broiler litter extracts, 10 200 to 11 000 for swine manure extracts, and 11 000 to 14 000 for cattle manure extracts. The relatively long delay time used here (5 s) allowed sufficient spin–lattice relaxation between scans for P compounds in these extracts with low paramagnetic ion concentrations (see Discussion). This was determined for selected samples by acquiring a set number of scans at different delay times up to 10 s.

Temperature was regulated at 20°C to minimize degradation of P compounds and ensure consistent signal intensities (Cade-Menun et al., 2002; Turner et al., 2003a). To allow comparison among extracts of each manure type, spectra were plotted using the line broadening necessary to produce acceptable resolution for the least-resolved spectrum of the three different NaOH concentrations. These values were 0.5 Hz for the broiler litter extracts, 1 Hz for the swine manure extracts, and 4 Hz for the cattle manure extracts. Where these values

concealed resolution, additional spectra were plotted using less line broadening.

Chemical shifts of signals were determined in ppm relative to 85% H_3PO_4 and assigned to individual P compounds or functional groups based on literature reports (Turner et al., 2003a). Signal areas were calculated by integration and P concentrations calculated by multiplying the proportion of the total spectral area assigned to a specific signal by the total P concentration (mg P g^{-1} dry manure) in the original extract. In well-resolved spectra, concentrations of phytic acid were determined by the same procedure by summing the areas of the four signals at approximately 5.95, 5.06, 4.70, and 4.54 ppm occurring in the ratio 1:2:2:1. In spectra where signals from phytic acid overlap with those from other monoesters, phytic acid concentrations can be calculated by multiplying the signal from the phosphate at the C-2 position on the inositol ring (occurring at approximately 5.95 ppm) by six, because this signal is often well-resolved from the orthophosphate and other monoester signals (O'Neill et al., 1980). Therefore, phytic acid concentrations were also calculated using this technique for comparison. Mean polyphosphate chain lengths were calculated from the concentrations of end groups (approximately −4 ppm) and mid-chain groups (−18 to −21 ppm) using the formula:

$$\text{mean chain length} = 2 + 2\left(\frac{\text{mid-chain groups}}{\text{end-chain groups}}\right)$$

It is difficult to estimate the error in NMR spectroscopy without acquiring replicate spectra, but for manure and feed samples, analytical error has been estimated to be approximately 5% for larger signals and 10% for smaller signals (Leinweber et al., 1997; Kemme et al., 1999). Differences in extraction efficiency with time and solution chemistry were investigated using analysis of variance procedures in SAS Version 8.0 (SAS Institute, 1999).

RESULTS

Phosphorus Recovery from Manures

Effect of EDTA on Phosphorus Recovery

Extraction solutions containing EDTA recovered a much greater proportion of total P than either NaOH alone or 1 *M* HCl (Table 2). Notably, NaOH–EDTA increased the recovery of the unreactive P fraction in swine and cattle manures compared with extraction with HCl. At least 25 m*M* EDTA was required for maximum recovery of total P, which was true of all NaOH concentrations (e.g., for 0.25 *M* NaOH; Fig. 1). Compared with NaOH alone, the inclusion of EDTA significantly increased the recovery of the reactive P fraction from cattle manure, and increased the recovery of both reactive and unreactive P by similar proportions in the swine manure and broiler litter (data not shown). The inclusion of EDTA also increased the recovery of Al, Ca, Fe, and Mn (e.g., for Ca and Fe; Fig. 1), although only relatively small proportions of Al, Fe, and Mn were recovered in more concentrated NaOH solutions (Table 3). To ensure an optimal concentration of EDTA in the extraction solution, 50 m*M* EDTA was used in all further experiments.

Table 2. Comparison of P recovery from animal manures in three different extraction solutions.†

	HCl‡	NaOH§	NaOH–EDTA¶
	% total P recovered		
Broiler litter	93 ± 3 (62)	35 (51)	96 ± 1 (63)
Cattle manure	63 ± 2 (23)	32 (78)	80 ± 2 (41)
Swine manure	82 ± 5 (6)	53 (18)	95 ± 1 (13)

† Values are means ± standard deviations of three replicate extracts (except for the NaOH extractions, for which single replicates only were extracted). Values in parentheses are the proportions of organic P in the extracts determined as the difference between total P (inductively coupled plasma–atomic emission spectrometry [ICP–AES]) and reactive P (molybdate colorimetry).
‡ Extracted for 1 h in 1 *M* HCl.
§ Extracted for 4 h in 0.25 *M* NaOH.
¶ Extracted for 4 h in 0.25 *M* NaOH and 50 m*M* EDTA.

Effect of NaOH Concentration on Phosphorus Recovery

Given a constant concentration of 50 m*M* EDTA, more P was recovered from cattle and swine manure in stronger NaOH solutions than in more dilute solutions ($P < 0.05$), although there was no significant difference

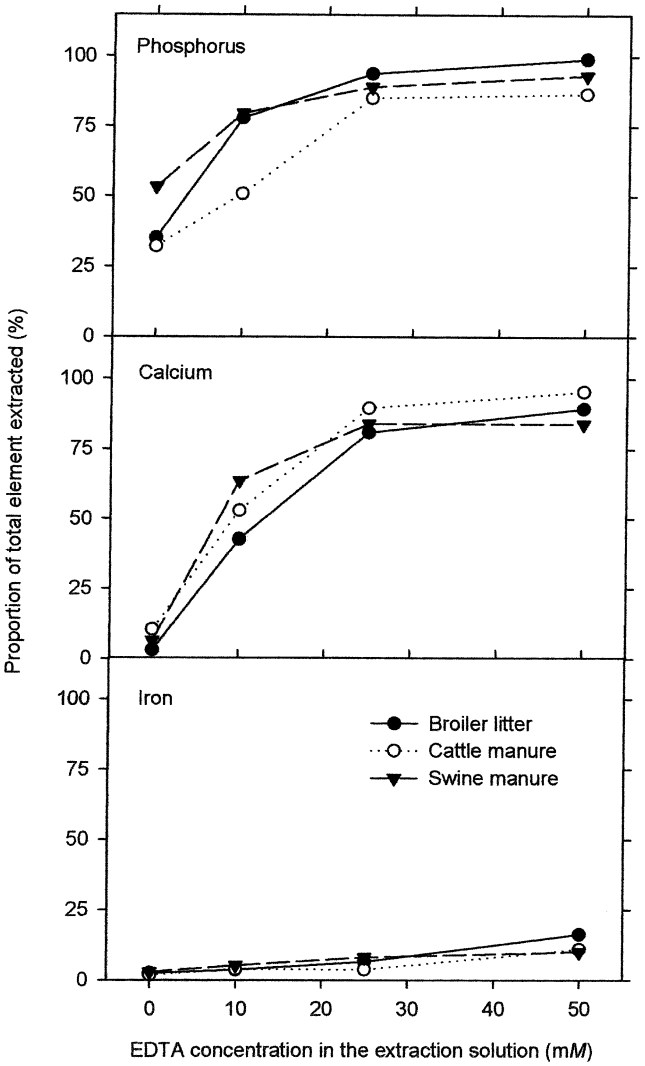

Fig. 1. Phosphorus recovery (%) from animal manures in alkaline extracts containing 0.25 *M* NaOH and varying concentrations of EDTA (m*M*). Manures were extracted for 16 h at 20°C.

Table 3. Recovery of operationally defined P fractions and cations from animal manures using three extractant solutions containing different concentrations of NaOH with constant 50 m*M* EDTA.†

NaOH molarity	Total P	Reactive P	Unreactive P	Al	Ca	Fe	Mn	pH‡
	mg P g^{-1} dry manure							
Broiler litter								
0.15 *M* NaOH	15.39 ± 0.20 (96)	5.68 ± 0.09 (36)	9.71 ± 0.16 (61)	0.27 ± 0.01 (56)	17.8 ± 0.3 (86)	0.55 ± 0.01 (64)	0.44 ± 0.01 (83)	10.7 ± 0.02
0.25 *M* NaOH	15.33 ± 0.20 (96)	5.76 ± 0.20 (36)	9.57 ± 0.23 (60)	0.22 ± 0.01 (46)	17.4 ± 0.5 (85)	0.16 ± 0.01 (19)	0.39 ± 0.01 (73)	11.6 ± 0.01
0.50 *M* NaOH	15.46 ± 0.19 (97)	5.76 ± 0.10 (36)	9.69 ± 0.24 (61)	0.04 ± 0.01 (8)	17.2 ± 0.1 (83)	0.04 ± 0.01 (5)	0.13 ± 0.01 (24)	12.2 ± 0.01
LSD (5%)	0.40	0.28	0.43	0.01	0.61	0.01	0.01	0.03
Cattle manure								
0.15 *M* NaOH	4.05 ± 0.08 (82)	2.49 ± 0.06 (50)	1.56 ± 0.02 (31)	0.05 ± 0.01 (3)	14.3 ± 0.5 (90)	0.15 ± 0.01 (13)	0.19 ± 0.01 (86)	10.5 ± 0.01
0.25 *M* NaOH	4.20 ± 0.08 (85)	2.49 ± 0.01 (50)	1.71 ± 0.08 (35)	0.08 ± 0.01 (5)	13.7 ± 0.3 (86)	0.14 ± 0.01 (12)	0.18 ± 0.01 (83)	11.2 ± 0.03
0.50 *M* NaOH	4.46 ± 0.04 (90)	2.56 ± 0.04 (52)	1.90 ± 0.02 (38)	0.07 ± 0.01 (5)	14.3 ± 0.3 (90)	0.06 ± 0.01 (5)	0.08 ± 0.01 (37)	12.1 ± 0.01
LSD (5%)	0.15	0.12	0.10	0.02	0.77	0.01	0.01	0.03
Swine manure								
0.15 *M* NaOH	12.66 ± 0.09 (87)	10.87 ± 0.38 (74)	1.79 ± 0.15 (12)	0.01 ± 0.01 (3)	10.0 ± 0.2 (85)	0.22 ± 0.01 (20)	0.07 ± 0.01 (63)	10.1 ± 0.03
0.25 *M* NaOH	12.86 ± 0.15 (88)	11.20 ± 0.25 (77)	1.67 ± 0.16 (11)	0.02 ± 0.01 (9)	10.2 ± 0.1 (86)	0.14 ± 0.01 (13)	0.07 ± 0.01 (66)	11.1 ± 0.01
0.50 *M* NaOH	13.40 ± 0.18 (92)	11.12 ± 0.09 (76)	2.27 ± 0.27 (16)	0.03 ± 0.01 (16)	9.9 ± 0.1 (84)	0.03 ± 0.01 (2)	0.03 ± 0.01 (31)	11.8 ± 0.01
LSD (5%)	0.23	0.27	0.50	0.01	0.26	0.01	0.01	0.03

† Values are means ± standard deviations of three replicate extracts, and values in parentheses are the proportions (%) of the total manure element recovered. Samples were extracted for 4 h at 20°C. See Materials and Methods for description of reactive and unreactive P fractions.
‡ Determined in the extraction solution following a 20-fold dilution.

in P recovery from broiler litter ($P > 0.05$; Table 3). In all manures, the changes in total P were accounted for by increased recovery of unreactive P, with little difference in reactive P concentrations (Table 3). Recovery of Fe and Mn decreased with increasing NaOH concentrations in all manures ($P < 0.001$), but Ca concentrations were not significantly affected ($P > 0.05$). Recovery of Al decreased in more concentrated NaOH extracts of broiler litter ($P < 0.001$), but increased in extracts of swine manure ($P < 0.001$) and was not significantly different in extracts of cattle manure ($P > 0.05$). Extract pH was more alkaline in stronger NaOH solutions, reflecting the extent of buffering in each manure type (Table 3).

Effect of Extraction Time on Phosphorus Recovery

Most total P in the manures was extracted after 1 h, although maximum recovery occurred between 4 and 16 h (e.g., for 0.5 *M* NaOH + 50 m*M* EDTA; Fig. 2). The differences in recovery between the 4- and 16-h extraction times were not significantly different ($P > 0.05$), so a 4-h extraction time was selected for all further experiments to minimize degradation of alkali-labile P compounds. The increase in P recovery during longer extraction times was mainly accounted for by the unreactive P fraction, with most of the reactive P being recovered during the first hour (data not shown). Calcium recovery followed a similar trend to P, but there were no significant increases in the recovery of Al, Fe, or Mn with increasing extraction time (data not shown).

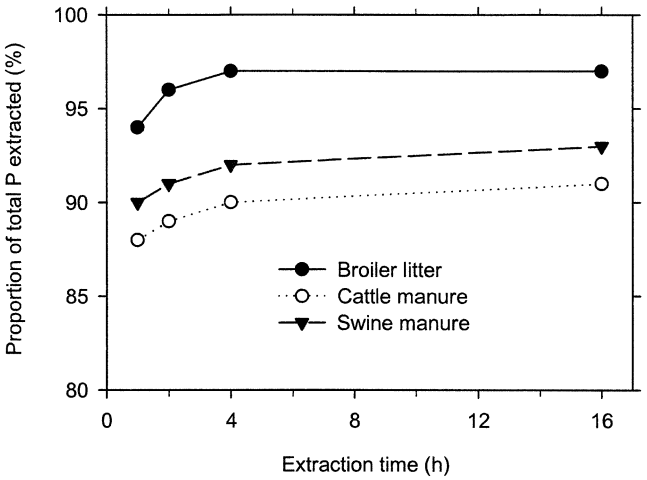

Fig. 2. Effect of extraction time on P recovery (%) from animal manures in 0.5 *M* NaOH + 50 m*M* EDTA.

Phosphorus Characterization by Solution Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy

Identification of Phosphorus Compounds

Spectra of 0.25 *M* NaOH + 50 m*M* EDTA extracts of the three manures are shown for comparison in Fig. 3, while expanded spectra of extracts of different NaOH concentrations are shown in Fig. 4, 5, and 6. Signals with similar chemical shifts were detected in the three manure extracts. A strong signal appearing between 6.17 and 6.28 ppm was assigned to inorganic orthophosphate, while signals between 4.0 and 6.0 ppm were assigned to orthophosphate monoesters. A number of individual signals were detected within this region, including strong signals at approximately 5.95, 5.25, 5.06, 4.90, 4.70, 4.54, and 4.40 ppm. Of these, the four signals occurring at 5.95, 5.06, 4.70, and 4.54 ppm in the ratio 1:2:2:1 were assigned to phytic acid, although these were only clearly resolved in the more concentrated NaOH extracts of swine manure and broiler litter. Some of the other signals in this region probably represented lower inositol phosphate esters, especially in extracts of the swine manure. Signals between 0.50 and 1.90 ppm were assigned to phospholipids, specifically phosphatidyl ethanolamine (approximately 1.8 ppm) and phosphatidyl serine (approximately 1.5 ppm). Signals between 0 and 1 ppm may originate from phosphatidyl choline or RNA, although both these compounds degrade rapidly in alkaline solution (Makarov et al., 2002; Turner et al., 2003a).

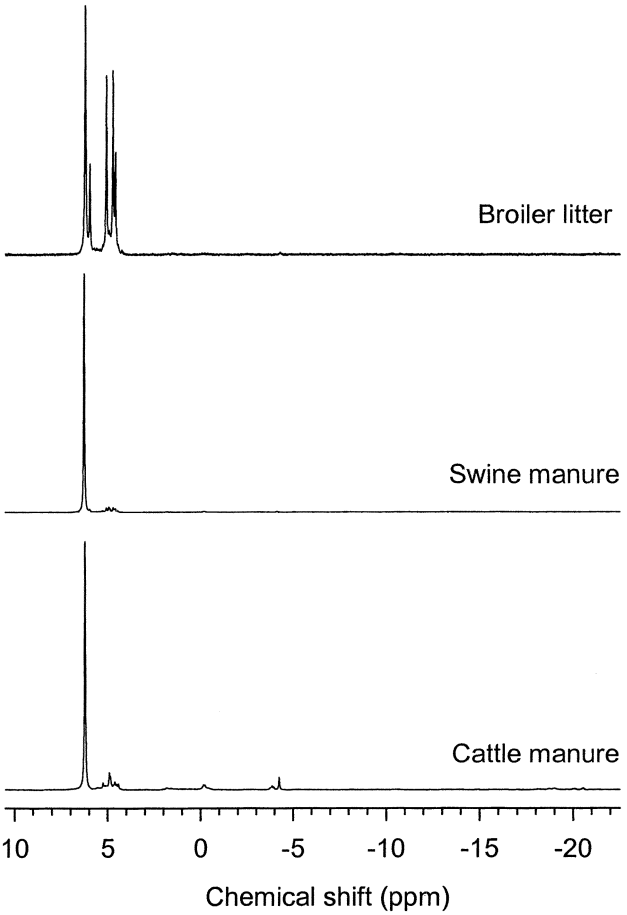

Fig. 3. Comparison of solution ^{31}P nuclear magnetic resonance (NMR) spectra of 0.25 *M* NaOH + 50 m*M* EDTA extracts of the three manures. Spectra are plotted using line broadening of 0.5 Hz (broiler litter), 1 Hz (swine manure), and 4 Hz (cattle manure).

Signals close to −0.3 ppm were assigned to DNA, while clear signals at approximately −4.3 ppm were assigned to pyrophosphate, a specific inorganic polyphosphate with chain length $n = 2$. Signals from longer-chain inorganic polyphosphates were detected between −3.8 and −4.0 ppm (end groups) and between −18 and −21 ppm (penultimate and mid-chain groups). Organic polyphosphates and phosphonates were not detected in any manure extracts.

Effect of NaOH Concentration on Spectral Resolution

The NaOH concentration of the extracts markedly influenced the spectral resolution of broiler litter and swine manure extracts (Fig. 4 and 5). Spectra of 0.15 *M* NaOH + 50 m*M* EDTA extracts were poorly resolved and exhibited considerable line-broadening, to the extent that signals from orthophosphate and the C-2 phosphate of phytic acid were inseparable. However, spectra of 0.50 *M* NaOH + 50 m*M* EDTA extracts were remarkably well-resolved, with signals in the monoester region clearly separated into distinct peaks. Line broadening was intermediate for 0.25 *M* extracts.

For the cattle manure, line broadening was similar for the different NaOH concentrations, although resolution was slightly improved in the 0.15 *M* NaOH + 50 m*M* EDTA extract (Fig. 6). For example, this was the only cattle manure extract in which the C-2 phosphate signal from phytic acid was clearly visible.

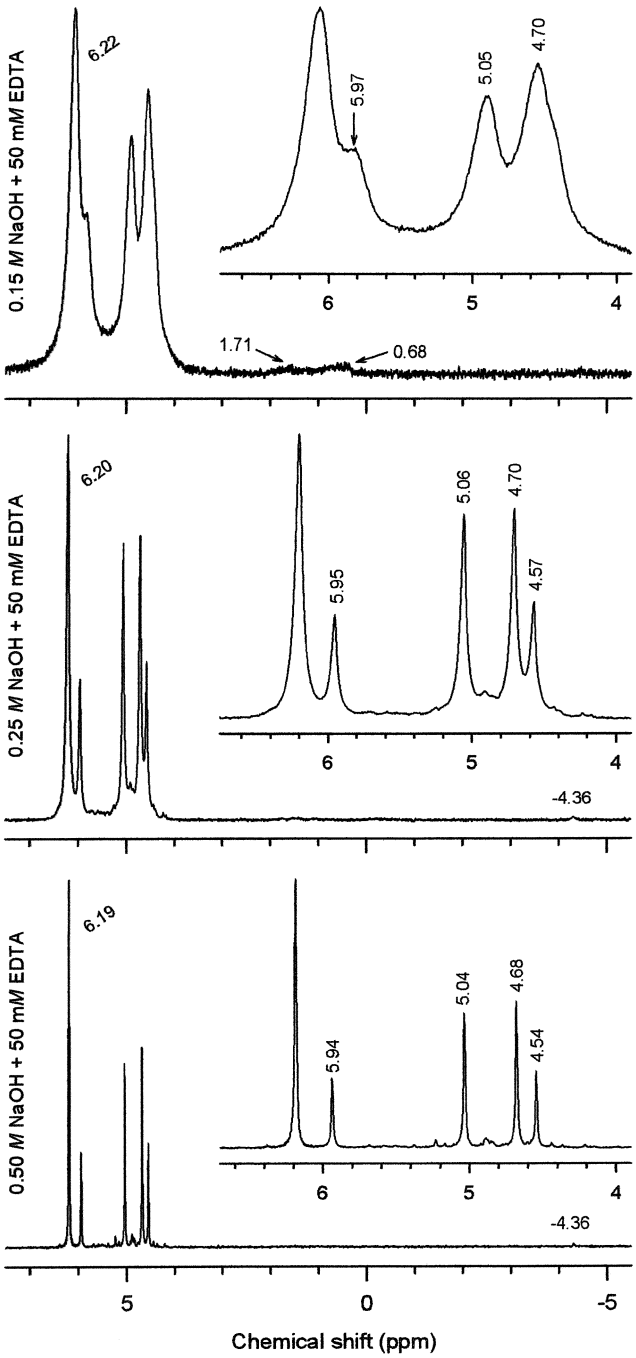

Fig. 4. Solution ^{31}P nuclear magnetic resonance (NMR) spectra of alkaline extracts of broiler litter extracted with different concentrations of NaOH (0.15, 0.25, and 0.50 *M*), and a constant concentration of 50 m*M* EDTA. All spectra are plotted using a line broadening of 0.5 Hz and scaled to the full height of the orthophosphate signal.

Differences in Phosphorus Composition between Manure Types

The three manures differed in their P composition (Table 4, Fig. 3). The broiler litter contained mainly

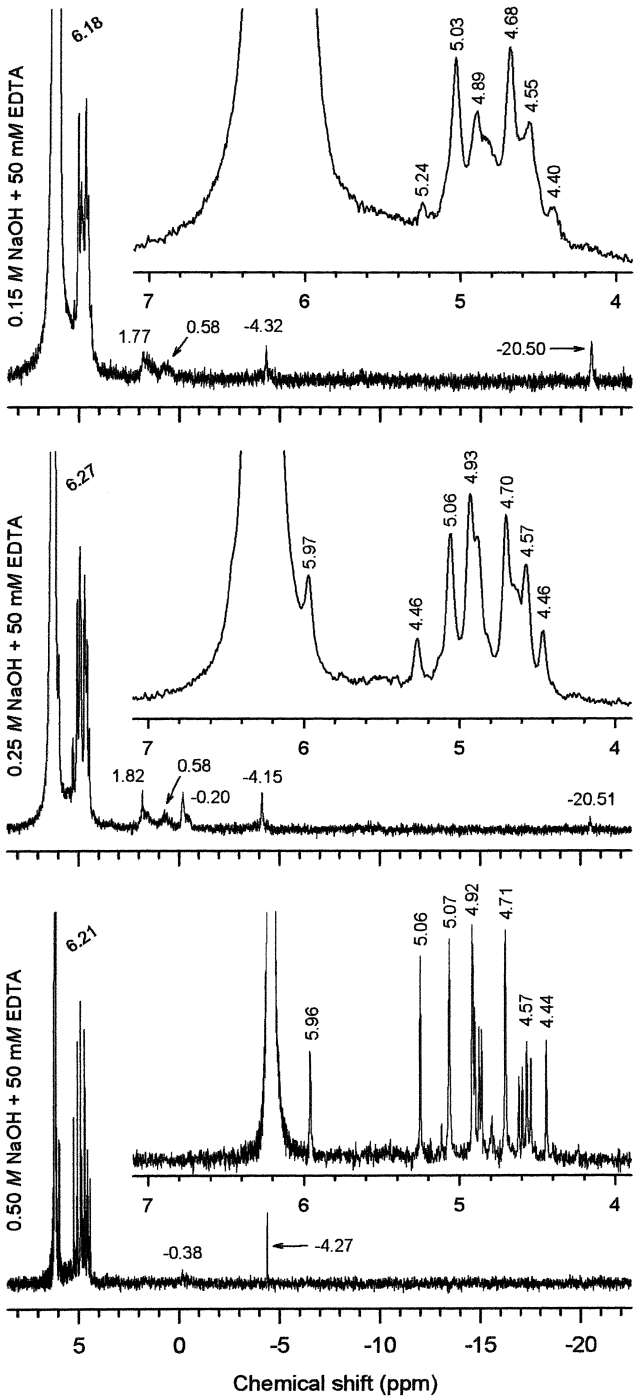

Fig. 5. Solution ^{31}P nuclear magnetic resonance (NMR) spectra of alkaline extracts of swine manure extracted with different concentrations of NaOH (0.15, 0.25, and 0.50 *M*), and a constant concentration of 50 m*M* EDTA. All spectra are plotted using a line broadening of 1.0 Hz, except for the inset spectrum of the 0.50 *M* NaOH + 50 m*M* EDTA extract, which was plotted using a line broadening of 0.3 Hz to preserve the enhanced resolution obtained for this spectrum. The main spectra are scaled to show the largest orthophosphate monoester signal as 75% of the full height of the figure.

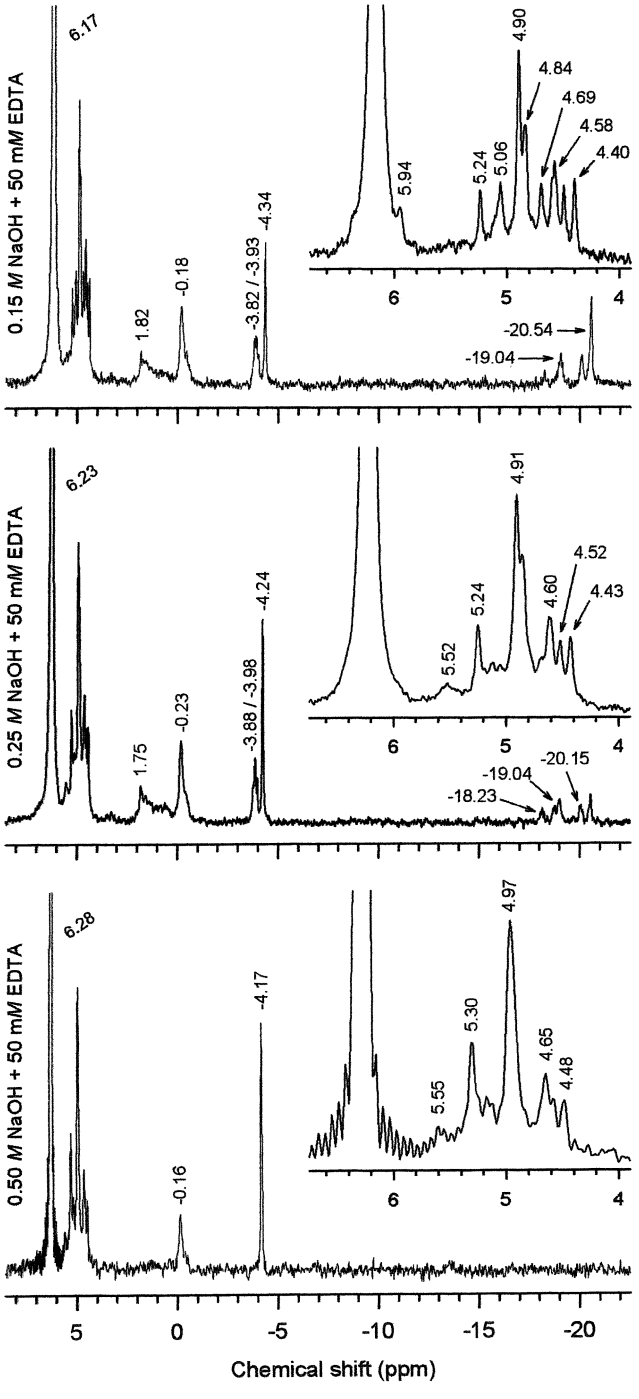

Fig. 6. Solution ^{31}P nuclear magnetic resonance (NMR) spectra of alkaline extracts of cattle manure extracted with different concentrations of NaOH (0.15, 0.25, and 0.50 *M*), and a constant concentration of 50 m*M* EDTA. The main spectra are plotted using a line broadening of 4 Hz, while inset spectra are plotted using line broadening of 1 Hz for the 0.15 *M* NaOH + 50 m*M* EDTA extract, 2 Hz for the 0.25 *M* NaOH + 50 m*M* EDTA extract, and 4 Hz for the 0.50 *M* NaOH + 50 m*M* EDTA extract. The main spectra are scaled to show the largest orthophosphate monoester signal as 75% of the full height of the figure.

orthophosphate and orthophosphate monoesters, with only traces of phospholipids and pyrophosphate, and no detectable polyphosphates or DNA (Fig. 4). In contrast, the cattle and swine manures were dominated by orthophosphate (Fig. 3). Cattle manure contained the richest P composition, including considerable proportions of orthophosphate monoesters, orthophosphate

Table 4. Phosphorus functional classes in alkaline extracts of animal manures using three extractant solutions containing different concentrations of NaOH with constant 50 m*M* EDTA.†

NaOH molarity	Orthophosphate	Orthophosphate monoesters	Orthophosphate diesters	Pyrophosphate	Polyphosphate
	mg P g^{-1} dry manure				
			Broiler litter		
0.15 *M* NaOH	7.47 (48.6)‡	7.21 (46.9)	0.70 (4.5)§	trace	ND
0.25 *M* NaOH	5.22 (34.1)	10.11 (65.9)	trace	trace	ND
0.50 *M* NaOH	6.12 (39.6)	9.34 (60.4)	trace	trace	ND
			Cattle manure		
0.15 *M* NaOH	2.73 (67.4)‡	0.59 (14.6)	0.28 (6.9)¶	0.11 (2.7)	0.34 (8.4)#
0.25 *M* NaOH	2.73 (65.0)‡	0.64 (15.2)	0.44 (10.5)††	0.16 (3.8)	0.23 (5.5)
0.50 *M* NaOH	2.92 (65.5)‡	1.04 (23.3)	0.19 (4.4)	0.30 (6.8)	ND
			Swine manure		
0.15 *M* NaOH	11.39 (90.0)‡	1.04 (8.2)	0.16 (1.2)§	0.03 (0.3)	0.04 (0.3)
0.25 *M* NaOH	11.20 (87.1)	1.32 (10.3)	0.29 (2.2)	0.05 (0.4)	0.01 (<0.1)
0.50 *M* NaOH	12.12 (90.4)	1.24 (9.3)	trace	0.04 (0.3)	ND

† Values in parentheses are the proportions (%) of the total P assigned to each fraction. Samples were extracted for 4 h at 20°C. ND, not detected.
‡ Includes the signal from the C-2 phosphate of phytic acid.
§ All orthophosphate diesters accounted for by phospholipids.
¶ Phospholipids, 0.09 mg P g^{-1} manure (2.1% total P); DNA, 0.19 mg P g^{-1} manure (4.8% total P).
# The ratio between polyphosphate signals at approximately −4 ppm (2.7% total P) and −18 to −21 ppm (5.7% total P) suggested an average chain length of $n = 6.2$, but this decreased to $n = 4.8$ in the 0.25 *M* NaOH extraction (see Materials and Methods for explanation of the calculation).
†† Phospholipids and DNA constituted equal proportions of the total orthophosphate diesters.

diesters (both DNA and phospholipids), and polyphosphates (both pyro- and polyphosphate). Indeed, these were the only extracts in which polyphosphate endgroups were visible (Fig. 6). Swine manure also contained orthophosphate monoesters, orthophosphate diesters, and traces of pyro- and polyphosphates. The high P concentrations in these extracts meant that most of the lower-concentration compounds were clearly detectable in the spectra (Fig. 5).

For the 0.50 *M* NaOH + 50 m*M* EDTA extracts of swine manure and broiler litter, spectra were so well-resolved that the four signals from phytic acid appeared as isolated peaks, allowing accurate identification and quantification of the phytic acid component (Table 5). Phytic acid concentrations and proportions calculated by the sum of all signals from phytic acid were much greater in the broiler litter (9.07 mg P g^{-1} manure; 59% total P) than the swine manure (0.67 mg P g^{-1} manure; 5% total P). Similar concentrations were obtained when calculated using the C-2 phosphate signal alone (Table 5).

Effect of NaOH Concentration on Phosphorus Composition

Phosphorus compounds detected by solution ^{31}P NMR spectroscopy varied when manures were extracted with different NaOH concentrations. In particular, some compounds detected in 0.15 *M* NaOH + 50 m*M* EDTA extracts were absent in spectra of more concentrated NaOH extracts, indicating that they had either degraded, or were not extracted, in the stronger alkaline solutions. For example, signals from phospholipids (0 to 2 ppm) and polyphosphates (−18 to −21 ppm) were completely absent from spectra of 0.50 *M* NaOH + 50 m*M* EDTA extracts of the cattle and swine manures, despite being significant components in 0.15 *M* NaOH + 50 m*M* EDTA extracts (Fig. 5 and 6). Changes in phospholipid signals were greatest between the 0.25 *M* and 0.50 *M* NaOH extracts, with much smaller changes between 0.15 *M* and 0.25 *M* NaOH extracts. However, some loss of polyphosphates was evident between the latter extracts of the swine and cattle manures associated with a reduction in mean chain length from $n = 6.2$ in the 0.15 *M* NaOH + 50 m*M* EDTA extract to $n = 4.8$ in the 0.25 *M* NaOH + 50 m*M* EDTA extract (Table 4). Changes in P composition were less evident in broiler litter extracts, because polyphosphates were not detected, and phospholipids were present only in trace amounts (Table 4, Fig. 4).

Signals from DNA, pyrophosphate, and orthophosphate monoesters did not change with increasing NaOH concentrations (Table 4; Fig. 4, 5, and 6), although there appeared to be some loss of the orthophosphate monoester signals at 4.69 and 5.06 ppm in the 0.25 and 0.5 *M* NaOH extracts of cattle manure (Fig. 6). Interestingly, DNA was apparently not extracted from swine manure by 0.15 *M* NaOH + 50 m*M* EDTA, only appearing in the spectrum of the 0.25 *M* NaOH + 50 m*M* EDTA extract (Fig. 5).

Table 5. Identification and quantification of the phosphate moieties of phytic acid in broiler and swine manure extracted in 0.5 *M* NaOH + 50 m*M* EDTA. Phytic acid gives four signals in the ratio 1:2:2:1, corresponding to the positions of the P nuclei on the inositol ring (Turner et al., 2003a, 2003b). Phytic acid concentrations were calculated by two methods: (i) multiplying the value for the C-2 phosphate by six (O'Neill et al., 1980) and (ii) summing the four signals from phytic acid.†

	Broiler litter	Swine manure
	mg P g^{-1} dry manure	
C-2 (5.95 ppm)	1.51 (9.76)	0.10 (0.75)
C-4, C-6 (5.06 ppm)	3.01 (19.54)	0.23 (1.73)
C-1, C-3 (4.70 ppm)	3.03 (19.69)	0.23 (1.70)
C-5 (4.56 ppm)	1.53 (9.95)	0.11 (0.82)
	Total phytic acid	
C-2 signal × 6	9.02 (58.58)	0.60 (4.47)
Sum of signals	9.07 (58.68)	0.67 (5.00)

† Values in parentheses are the proportions (%) of the total extracted P represented by phytic acid.

DISCUSSION

Recovery of P from animal manures was clearly influenced by the extraction solution. The inclusion of EDTA allowed an alkaline extractant (required to ensure optimal spectral resolution and consistent chemical shifts for solution ^{31}P NMR spectroscopy) to recover >90% of the total P from the three manure types. This is a considerable improvement on the use of NaOH alone, which typically recovers <50% of the total manure P (Leinweber et al., 1997; Dou et al., 2000). A similar improvement in P extraction from plant litter and soils following inclusion of EDTA is probably due to chelation of Al and Fe compounds (Cade-Menun and Preston, 1996), whereas the increased P recovery from manures seems mainly due to increased recovery of Ca compounds, which are poorly soluble in alkaline solution.

The extraction solution also markedly influenced results from solution ^{31}P NMR spectroscopy, because the concentration of NaOH affected both spectral resolution and the apparent P composition of the manures. The only significant difference in solution chemistry among the different NaOH extracts was the concentration of paramagnetic ions, because almost no Fe and Mn were present in the 0.5 *M* NaOH + 50 m*M* EDTA extracts. This seems a likely explanation of differences in spectral resolution, because paramagnetic ions are known to influence line broadening, and even relatively small concentrations can reduce spectral quality (O'Neill et al., 1980). However, this does not account for the spectra of cattle manure extracts, because resolution was similar for all three NaOH concentrations, despite a decrease in paramagnetic ion concentration in the strongest NaOH extract. Spectral resolution can be influenced by sample pH (Crouse et al., 2000), but this was ruled out because the redissolved extracts analyzed here were all at pH of >13.7, which is sufficiently high to eliminate such effects. A more likely explanation is the more viscous nature of the cattle manure extract compared with the swine manure and broiler litter extracts, because increases in viscosity can also increase line broadening (Nanny et al., 1997). This phenomenon requires further investigation, because similar improvements in spectral resolution would significantly enhance the spectroscopic analysis of P in a range of environmental samples, notably soils and sediments.

Improvements in spectral resolution by extraction with more concentrated NaOH solution were compromised by the loss of signals from phospholipids and polyphosphates. These compounds were quantitatively unimportant in broiler litter, and to a lesser extent in the swine manure (Peperzak et al., 1959; Barnett, 1994), but constituted relatively large proportions of the total organic P in the cattle manure. Degradation of phospholipids was expected in the stronger alkaline solutions (Turner et al., 2003a) and was reported previously in swine slurry (Leinweber et al., 1997). However, all extracts were redissolved in 0.9 *M* NaOH for NMR spectroscopy, suggesting that any alkali-induced degradation should have been consistent across all samples irrespective of the strength of the initial extract. For polyphosphates, degradation is minimal in 0.25 *M* NaOH + 50 m*M* EDTA extracts of soils and sediments, because metal chelation by EDTA precludes chemical hydrolysis (Hupfer et al., 1995; Turner et al., 2003a). Therefore, changes in polyphosphate signals with increasing NaOH concentration are more likely to be explained by changes in solubility. In particular, the marked decreases in metal concentrations in stronger alkaline solutions indicate possible coprecipitation with polyphosphates. However, pyrophosphate concentrations in the more alkaline cattle manure extracts increased in proportion with decreases in polyphosphate concentrations (Table 4), suggesting that at least some degree of degradation was involved. It should be noted that significant quantities of polyphosphates have not previously been reported in manures, but their presence clearly demonstrates that simple classification of extractable P into inorganic and organic fractions based on molybdate colorimetry can be misleading.

A large difference in phytic acid concentrations was detected between swine manure and broiler litter, but this was not unexpected. Poultry litters typically contain considerable proportions of phytic acid (Barnett, 1994), while the value of 4.5 to 5% of the total P reported here for swine manure is similar to that of 4% reported by Kemme et al. (1999). However, despite the small concentrations of phytic acid in the swine manure, the well-resolved spectrum ensured that it was quantified with some confidence. Clearly, the NMR procedure is sensitive enough to detect low concentrations of organic P compounds (e.g., approximately 100 μg P g^{-1} dry manure), yet robust enough to achieve this in the complex matrices of manure extracts.

It would also be possible to quantify phytic acid in the 0.25 *M* NaOH extract of the broiler litter, but overlapping signals from phytic acid and orthophosphate or other monoester signals mean there is a much greater chance of introducing error. This problem can be overcome by calculating phytic acid concentrations using the signal from the C-2 phosphate of phytic acid (O'Neill et al., 1980). Phytic acid concentrations in the swine manure and broiler litter extracts were similar when calculated based on the sum of all phytic acid signals or the C-2 phosphate alone. This suggests that phytic acid can be quantified with confidence even in relatively poorly resolved spectra providing that signals from orthophosphate and the C-2 phosphate are sufficiently resolved.

None of the spectra of cattle manure extracts were sufficiently resolved in the orthophosphate monoester region to permit the quantification of phytic acid. This may be overcome by pretreating extracts with hypobromite oxidation before NMR spectroscopy (Irving and Cosgrove, 1981), or by using spectral deconvolution software to separate phytic acid signals from complex spectra (Turner et al., 2003b). However, the cattle manure appeared to contain only small concentrations of phytic acid, because the signal from the C-2 phosphate of phytic acid was small and only visible in the spectrum of the 0.15 *M* NaOH + 50 m*M* EDTA extract. This was not unexpected, because pasture plants contain little phytic acid compared with the grains fed to swine and

broilers (Peperzak et al., 1959). It was noted that signals from phytic acid appeared at chemical shifts slightly upfield of those measured in soil extracts (Turner et al., 2003a). Again, these were not due to differences in pH in the redissolved extracts, but may have been caused by differences in solution chemistry, because manure extracts contained large concentrations of Ca, but low concentrations of paramagnetic ions, compared with soil extracts.

Selection of an appropriate delay time is important to ensure quantitative analysis by allowing all P nuclei to fully relax between scans (Cade-Menun et al., 2002). In soil extracts, the high concentrations of paramagnetic ions allow relatively short delay times (<1 s) to be used, because the magnetic properties of Fe and Mn help P nuclei to relax more rapidly after excitation than would otherwise be expected if paramagnetics were not present (Wilson, 1987). This minimizes the machine time required to obtain acceptable signal-to-noise ratios. However, the low concentrations of paramagnetic ions in the manure extracts analyzed here necessitated relatively long delay times of at least 5 s for quantitative analysis, which meant that several hours of machine time were required to obtain suitable spectra. It may be possible to decrease this by adding small concentrations of lanthanide shift reagents or paramagnetic ions to the NMR tube to help P nuclei relax more rapidly (Nanny et al., 1997), although O'Neill et al. (1980) reported that there is a small and narrow range of optimum paramagnetic ion concentrations above which any advantage of decreased spin–lattice relaxation time is compromised by increased line-broadening.

CONCLUSIONS

Alkaline extraction and solution ^{31}P NMR spectroscopy provides a relatively simple and accurate procedure for the analysis of P compounds in animal manures. However, results are markedly influenced by the extractant, which affects both spectral resolution and the apparent P composition of the manures. The choice of extractant will therefore depend on the type of manure being analyzed and the specific objectives of the study. Quantification of phytic acid in swine manure and broiler litter will be best achieved by extraction with 0.50 *M* NaOH + 50 m*M* EDTA, although phytic acid cannot be quantified in cattle manure extracts using this procedure without additional treatment by hypobromite oxidation. For complete P characterization, or for comparison among different manure types, extraction with 0.25 *M* NaOH + 50 m*M* EDTA is likely to provide the optimum balance between spectral resolution and the detection of all P compounds.

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